Pesticidal character of phytoecdysteroids from *Ajuga multiflora* Bunge (Labiatae) on larvae of *Cryptorrhynchus lapathi* L. (Coleoptera: Curculionidae)

CHI De-fu[1], SUN Ming-xue[2], XIA Wen-fu[3]

([1]*College of Forest Resources and Environment, Northeast Forestry University, Harbin 150040, P.R. China)*

([2]*Forest Management Bureau, Great Xing'an Mountain, Jiagedaqi, 165000, P.R. China)*

([3]*Forest Bureau, Lindian, Heilongjiang Province, 163000, P.R. China)*

Abstract: Eight kinds of phytoecdysteroids extracted from different parts of *Ajuga multiflora* Bunge (Labiatae) that were collected from different places at different time were tested for killing effects on the 2-instar larvae of *Cryptorrhynchus lapathi* L by adding them to the artificial diet of larvae. The experimental results indicated that adding 1-3-mL phytoecdysteroids to the artificial diet could lead 58%-100% of 2-instar larvae of *C. lapathi* to death within 24 days. The phytoecdysteroids extracted from the whole plant *of A. multiflora* which was collected before flowering time were much more effective than those extracted from the plants collected at flowering and after flowering periods, and the modified mortality rate of larvae reached 65.22%, 85.07%, and 98.11% at the dosage level of 1-mL, 2-mL, and 3-mL extracts, respectively. The extract made from root of *A. multiflora* plant was more effective in killing efficiency than those from stem and leaves, and the average death rates of larvae were up to 100%, 98.20% and 98.32% at dosage levels of 1-mL, 2-mL, and 3-mL extracts, respectively. The killing speed of the extracted phytoecdysteroids was slower than that of triflumuron, hexaflumuron or deltamethrin emulsifiable concentrate. The mortality rate of larvae is closely related to the feeding duration on the diets containing phytoecdysteroids. Feeding on the diets with addition of phytoecdysteroids for 16 days, more than 80% of treated 2-instar larvae of *C. lapathi* were led to death. The killing effect of the extracts was little affected by the growth areas of *A. multiflora* plant and the adding way to artificial diet.

Keywords: *Ajuga multifora* Bunge; *Cryptorrhynchus lapathi* L.; Larva; Phytoecdysteroid

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Introduction

Phytoecdysteroids are a sort of active natural compounds that can regulate the growth of insect pests, and their chemical structures are similar with the molting pheromone secreted by insects. Phytoecdysteroids are containing in plants (Koreeda *et al*, 1972; Camps *et al*, 1985a, 1985b, 1990b; Nie *et al.* 1987; Qiu *et al.* 1997). More than 40 years ago, scholars began to study the composing and content of those compounds in different plants, as well as their physiological effect on different insects (Shimomura *et al*, 1970; Bandara *et al*, 1989; Bathory *et al*, 1990; Camps *et al*, 1990a; Chi *et al.* 1997a; Shao *et al.* 1987). Up to now, more than 130 kinds of phytoecdysteroids have been found. The most common compounds are 20-OH ecdysone, cyasterone, makisterone, ajugalactone, makisterone, *etc.* (Darvas *et al* 1997). These compounds not only can disturb the growth and molting of treated insects, but also can bring them to sterility even to death. Some of those phytoecdysones, at a certain dosage, acting like insect juvenile hormone, can make larvae develop into overage one, and some of them can induce insect into diapause (Camps, *et al.* 1990c, 1991; Darvas, *et al.* 1991). The pesticidal character of those phytoecdysteroids on some insect pests, such as *Clostera anastmosis* (Linnaeus), *Stilpnotia candida* (Staudinger), *Tuberolachnus salignus* (Gmelin), *Hyphantria cunea* (Drury), *Aporia crataegi* Linneaus, *Malacosoma neusteria testacea* Motschulsky, *Lymantria dispar* L., *Aphrophora intermedia* Uhler, *Parthenolecanium corni* (Bouche), *Myzus persicae* (Sulzer) etc, were also been studied (Chi *et al.* 1997a, 1997b; Darvas *et al.*1997, Shao 1987). High concentrated phytoecdysteroids can lead pests to death, while lower concentration usually lead insects to pupation, so it is widely used in China to make larvae of *Bombyx mori* (L.) pupating synchronously in later autumn (Nie *et al.* 1987).

Many plants contain phytoecdysteroids. More than 100 *Ajuga* species live in the world and 18 species distribute in China. Most of those *Ajuga* species contain phytoecdysones, for example, *Ajuga decumbens* Thunb, *A. nipponensis* Makino, *A. multiflora* Bunge and *A. inearifolia* Parmp. Among those species, *A. multiflora* Bunge, contains comparatively higher concentration of phytoendysones (Darvas *et al.* 1997). To understand the using potential of those Phytoecdysteroids contained in *A. multiflora* in the sustainable controlling of trunk borer, this paper made studies on the killing effects of those phytoecdysteroids on larvae of

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Biography: CHI De-fu (1962-), male, Ph D, professor in Northeast Forestry University, Harbin 150040, P R China E-mail: chidefu@0451 com

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Cryptorrhynchus lapathi L. (Coleoptera: Curculionidae).

Materials and methods

Materials

Indian meal, soybean flour, powder of popular bark, mixed vitamin liquor, Wesson salt, sodium benzoate, distilled water, and glass plate (15cm×15cm) were used in rearing larva of *C. Lapathi* L. 2-instar larvae of *C. lapathi* L. were collected on April 24, 1999, from poplar stands in Hongqi Forest Farm, Daqing city, Heilongjiang Province.

A. multiflora Bunge (Labiatae) plants used for makin powder and extracts of phytoecdysteroids were collected from Qianshan in Liaoning Province, Zhalantun in Inner Mongolia and Jiagedaqi in Great Xing'an Mountain area of Heilongjiang Province, before flowering, at flowering time, and after flowering. The collected plants were dried naturally in the shade.

Five percent hexaflumuron emulsifiable concentrate (HEC) was provided by Dalian Razer Pesticides Co., Ltd; 5% triflumuron emulsifiable concentrate (TEC) was provided by Jilin Tonghua Pesticide & Chemical Stock Co., Ltd.; and 2.5% deltamethrin emulsifiable concentrate (DEC) was bought from market.

Methods

Rearing larvae.

C. Lapathi L is a kind of boring pest. Younger larva bores between phloem and xylem and eats cambium and older larva digs tunnels into the xylem. It is almost impossible to rear this pest on a segment of trunk and observe its reaction to pesticides indoor. Therefore, an artificially rearing method might be adopted. In our experiments, an artificial diet used by Hou Aiju (1991) was adopted but some modifications were made, which is the most suitable for the growth of those larvae (see Table 1).

Table 1. Main components in the artificial diet for the larvae of *Cryptorrhynchus lapathi* L

Main components	Dosage	Main components	Dosage
Indian meal (g)	20	Wesson salt (g)	0.5
Soybean flour (g)	4	Sodium benzoate (ml)	0.5
Powder of poplar bark (g)	8	Distilled water (ml)	20
Mixed vitamin liquor (g)	0.5		

Note: 1) Mixed vitamin solution are 100-mg nicotinic acid, 100-mg partothenic acid, 50-mg riboflavin, 25-mg pyridoxine, 25-mg thiamire, and 25-mg folocin, dissolved in 1000-mL water; 2) Wesson salt: 10.00 g $CaCo_3$, 0.039 g $CuSO_4 \cdot 5H_2O$, 1.47g $Fe_3(PO4)_2 \cdot 2H_2O$, 0.02g $MnSO_4$, 5.00g $MgSO_4$, 0.009g $KAl(SO_4) \cdot 2H_2O$, 6.00g KCl, 16.00g KH_2PO_4, 0.005g KI, 0.057g NaF and 6.00g NaCl dissolved in 1000-mL water; 3) Sodium benzoate solution is 0.6 sodium benzoate dissolved in 1000ml water; 4) Powder of poplar bark is the dried fresh popular bark in the air, then crush it into fine powder.

After blending ingredients uniformity in a glass container, this artificial diet was put between two pieces of glass plate. This food is about 0.4-0.5 cm in thickness. It was steamed in a steamer and cooled naturally. After cooling, a small hole was made in one side of a piece of diet, after then we put larvae into these holes (each hole for one larva, or each piece of diet only reared one larva) and let larvae feed on the artificial diet. Breeding plates were kept in an animal rearing room at constant temperature of 25-28°C, under relative humidity of 60%-70% and dark condition. Whenever these diets were decomposed or these tunnel were almost transfixion, diets were renewed and the living status of the larvae was recorded. Diets were usually renewed every five days.

Making phytoecdysteroids extracts from Ajugap plants

Naturally dried *A. multiflora* plant was grounded to powder. 20-g powder was weighed and put into a glass container, and 150-mL 100% methanol was added, after then it was sonicated for 5 min and centrifuged for 15 min at 2800rpm. Supernatant (methanol phase) was collected. The sediment was extracted again with 150-mL methanol. The supernatants obtained from the second extraction was mixed with first one. The mixed methanol phase was evaporated under vacuum. After solvent was removed, 20-mL ethanol was added to those dried extracts, and 1-mL methanol extracts could be obtained from one gram dried materials. These extracts were stored at a temperature of -50°C. Eight kinds of different extracts were obtained by this method. We give each of them an abbreviation so that we can use it easily in the discussion (see Table 2).

Table 2. Abbreviation of extracted phytoecdysteroids made from *Ajuga multiflora*.

Collecting place	Part of plants	Collecting time	Abbreviation of extracts
Qianshan	whole plant	Before flowering	QWBE
		Flowering	QWFE
		After flowering	QWAE
	root	Flowering	QRFE
	stem	Flowering	QSFE
	leaves	Flowering	QLFE
Zhalantun	Whole plant	Flowering	ZWFE
Jiagedaqi	Whole plant	Flowering	JWFE

Effect of phytoecdysteroids on larvae of C. lapathi L.

The pesticidal activity of those phytoecdysteroids was tested with consideration of the influences of collecting time of plants, growing place, or different part of the plant. Those extracts, QWBE, QWFE, QWAE, QRFE, QSFE, QLFE, ZWFE, and JWFE, were taken out from the refrigerator and sonicated two times (30s each) to make the extracts solve sufficiently. The artificial diet was added with one of the extracts at a dosage of 1 mL, 2 mL, and 3 mL in company with adding water. At the same time, we set 1-mL, 2-mL, and 3-mL 5% HEC, 5% TEC, and 2.5% DEC treated diets as comparison. The diet with addition of 3-mL distilled water was taken as contrast group. We added the extracts to the diets at each time the diets were renewed. Three repetitions

were set for each treatment. Fifteen to twenty larvae were treated in each repetition.

For testing the difference in mortality rate of larvae caused by treating periods, 3-mL sonicated QWFE, 3-mL 5%HEC, 3-mL 5%TEC, and 3-mL 2.5% DEC was added to the artificial diet in company with adding water, and the diet with addition of 3-mL distilled water was set as contract group. Six different treated periods, 4, 8, 12, 16, 20, and 24 days, were arranged. Three repetitions were set for each treatment and 15-20 heads of larvae were treated in each repetition. Living status of larvae was observed and recorded every 2 days. Totally 24 days was observed.

The difference in the mortality rate modified by the ways of adding phytoecdysteroids to diets was studied. Three ways were used in terms of adding those phytoecdysteroids. The first way, in company with adding water, was to add 1-mL, 2-mL, and 3-mL QWFE to diet, then those diets were steamed and larvae were inoculated into it. The second way was to dilute 1-mL, 2-mL or 3-mL QWFE in 10-mL water and separately injected them into a cooked diet slowly using injector. These moist diets were put in a shady room to let water evaporate naturally. When the water content of diet dropped down to just cooked level, larva was introduced into it. The third way was to add 1, 2, and 3 g of *Ajuga* powder to the diet before it was cooked. This powder was made from the whole plant that was collected from Qianshan area at flowering time. Then this diet was cooked and larva was inoculated into it. Diet with addition of distilled water was used as contrast. Three repetitions were set for each treatment and 15 to 20 larvae were treated in each repetition. Experiment was observed and recorded every 2 days. Diet was replaced by newly cooked one every 5 days.

Results and analysis

Influence of growing place, collecting time or part of plant

On April 24, 1999, trunk segments were carried back to laboratory of Northeast Forestry University. Larvae of *C. lapathi* L. were taken out from those dissected trucks and put into the holes made in the artificial diet. Living status of those larvae was checked every 48 h. Diets were renewed 4 times within 24 experimental days. Data recorded from this experiment was counted and listed in Table 3.

From those data listed in Table 3, we found that the phytoecdysteroids extracted from *Ajuga* plants have pesticidal activities. One to three milliliters of those extracts led 58% to 100% of the *C. lapathi L.* larvae to death. Among the 8 kinds of extracts used, QRFE was the most efficient one. The modified mortality rates of the 2-instar larvae that fed on the diets with addition of 1-mL, 2-mL, and 3-mL QRFE were up to 100%, 98.20% and 98.32% respectively. This results was similar with that led by 5% HEC, 5% TEC or 2.5% DEC in same dosage.

The data from QWBE, QWFE, QWAE, ZWFE, JWFE treated groups showed that the mortality rates of the treated larvae were positively related with the dosage of extracts added in the diet. A higher dosage of phytoecdysteroids induced higher mortality rate. For example, when the diets were separately treated with 1-mL, 2-mL and 3-mL QWBE, correspondingly 66.22%, 85.07% and 98.11% of treated larvae were caused to death.

In comparison with QWBE, QWFE and QWAE, which were all made from whole plant but the plants were collected in different time (see Table 2), QWBE (before flowering) was most effective in killing 2-instar larvae of *C. lapathi L*, and the QWAE (after flowering) was comparatively inefficacy. The extracts QWAE, in terms of death rate of treated larvae, was 9.78%, 25.45% and 23.59% lower than QWBE. The killing effectiveness of QWFE (at flowering time) was in the middle among the three kinds of extracts. This result showed that extracts made from *A. multiflora* plants collected before flowering was more effective than those extracts made from plants at flowering time or after flowering.

Comparison of QRFE (root), QSFE (stem) and QLFE (leaf) treated groups showed that QRFE was most efficient in killing larvae of *C. lapathi L*, with a mortality rate of treated larvae of 100%, 98.20%, and 98.32% at a corresponding dosage of 1 mL, 2 mL, and 3 mL. The mortality rate of those larvae treated with QSFE or QLFE at the corresponding dosage was 8.41% to 22.75% lower than that treated by QRFE. This result indicated that the extracts made from the root of *A. multiflora* at flowering time was more effective than those made from stems or leaves.

The larvae treated with extracts QWFE, ZWFE and JWFE, which were all made from whole plant but collected from different areas at flowering time, had 66.67%, 57.88% and 62.17% mortality respectively at a dosage of 1 mL and 73.04%, 77.13% and 70.25% mortality respectively at a dosage of 2 mL. More than 88.24% of larvae died after fed on diet with addition of 3 mL of those extracts. The results of this comparison showed that those extracts made from *A. multiflora* L. collected from Qianshan, Zhalantun, or Jiagedaqi at flowering time have similarly pesticidal activity on the 2-instar larvae of *C. lapathi* L.

Killing rapidity of those phytoecdysteroids

In this experiment, 3-mL QWFE, 3-mL 5%HEC, 3-mL 5% TEC, and 3-mL 2.5% DEC were added into the diet of *C. lapathi* L. and their killing effects were separately tested. Twelve times was observed within 24 days. The living status of larvae was recorded every 48 h. The data recorded was calculated and plotted as Fig. 1. Fig. 1 showed that the killing speed of QWFE was much slower than that of HEC, TEC or DEC. The mortality rate of those larvae treated by QWFE was 0 in the first 6 days and was not over 50% in 16 d. The death rate of larvae got up to 98.44% in 24 d after treatment. The death rates of larvae were over 50% in 10 d in the HEC, TEC or DEC treated group. In the DEC treated group, 32.88% larvae died within 2 d, more than 50% died in 4 d and 100% died in 6 d.

Table 3. The mortality rate of larvae taken artificial diet containing phytoecdysteroids from *A. multiflora*

Extracts or pesticides	Dosage used (ml)	Repetition	Number of larvae treated	Number of dead larvae	Average mortality rate (%)	Modified mortality rate (%)
	1	3	62	42	67.74	65.22
QWBE	2	3	65	56	86.16	85.07
	3	3	57	56	98.25	98.11
	1	3	55	38	69.09	66.67
QWFE	2	3	56	42	75.00	73.04
	3	3	57	54	94.74	94.32
	1	3	55	34	61 82	58.84
QWAE	2	3	56	37	66.07	63.42
	3	3	56	43	76.79	74.97
	1	3	57	57	100.00	100.00
QRFE	2	3	60	59	98.33	98.20
	3	3	64	63	98 44	98.32
	1	3	63	54	85.71	84.60
QSFE	2	3	67	52	77.61	75.86
	3	3	54	49	90.74	90.02
	1	3	57	51	89.47	88.65
QLFE	2	3	58	52	89.66	88.85
	3	3	57	50	87.72	86.76
	1	3	64	39	60.94	57.88
ZWFE	2	3	66	52	78.79	77.13
	3	3	57	51	89.47	88.65
	1	3	57	37	64.91	62.17
JWFE	2	3	58	42	72.41	70.25
	3	3	55	49	89.09	88.24
5% triflumuron emulsifiable concentrate	1	3	59	54	91.53	90.86
	2	3	60	60	100.00	100.00
	3	3	57	57	100.00	100.00
5% hexaflumuron emulsifiable concentrate	1	3	63	63	100.00	100.00
	2	3	65	65	100.00	100.00
	3	3	64	64	100.00	100 00
2.5% deltamethrin emulsifiable concentrate	1	3	66	66	100.00	100.00
	2	3	62	62	100.00	100.00
	3	3	73	73	100.00	100.00
Water (contrast group)		5	124	9	7.26	

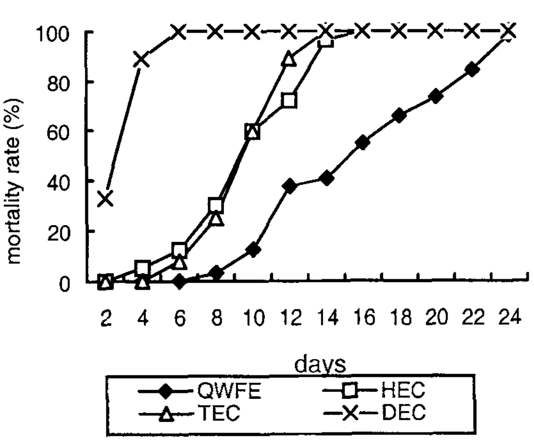

Fig 1. The comparison of the mortality speed of 2-instar larvae of *C. lapathi* treated by different chemicals

Time of period of those larvae fed on treated diet

This experiment was investigated every 48h in 24 days. The modified mortality rate of those larvae was calculated and shown as Fig. 2. The mortality rate varied evidently with the time (days) larvae fed on treated diet with phytoecdysteroids. After feeding on the treated diet for 4 d, 8 d and 12 d, correspondingly 12.79%, 37.91% and 37.58% of larvae died. After 16 d, the mortality rate of larvae increased rapidly, with a modified death rate of 87.24% at 16 d, 84.03% at 20 d and 94.32% at 24 d

The way of adding phytoecdysteroids into diet

The ways used to treat diet were as follows: (1) treated with extracted phytoecdysteroids before diet was braised (MQWFE); (2) treated with extracted phytoecdysteroids after diet was braised (SQWFE); (3) added *Ajuga* powder directly into the uncooked diet (MWFP). We calculated the modified death rates for the three treated groups and graphed them into Fig. 3. The results showed that these

ways used for adding phytoecdysteroids into artificial diet were effective in controlling of 2-instar larvae of *C. lapathi* L. There was no significant difference in mortality rate conducted by those kinds of treatment. For example, the modified death rates of larvae obtained by treatments of MQWFE, SQWFE and MQWFP were 66.67%, 68.79%, and 64.15% respectively under dosage of 1-mL (g) phytoecdysteroids and were up to 94.32%, 96.63%, and 88.14% under a dosage of 3 mL (g), respectively.

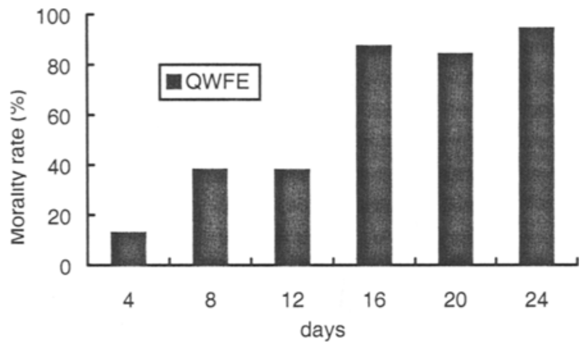

Fig. 2 Mortality rate of 2-instar larvae of *C. lapathi* L in different periods (days) after eaten phytoecdysteriods

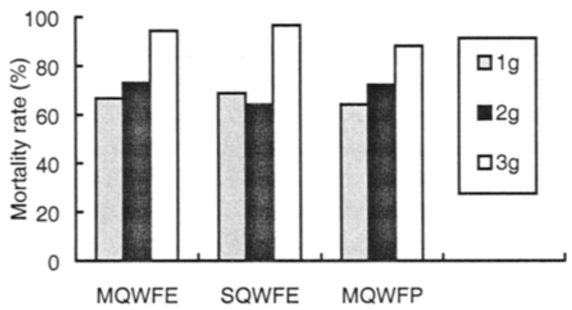

Fig. 3 Effects of different treatment methods of artificial diet on mortality rate of 2-instar larvae of *C. lapathi* L

Discussion

From this experiment, it was found that those extracts made from plants collected at varied time have different pesticidal abilities. The extracts made from the plants that were collected before flowering were more effective in killing of 2-instar larvae of *C. lapathi*, but those made from the plants collected after flowering were not so efficient. The modified mortality rate of 2-instar larvae who fed on 3-mL QWBE treated diet was up to 98.11%, but it was only 74.97% of the larvae to be killed for those feeding on the 3-ml QWAE treated diet. This result was most likely to be induced by the difference in contentment of phytoecdysteroids in plants varying with growth periods.

Testing results of the extracts made from different parts of flowering *A. multiflora* plant showed that the extracts obtained from root was more effective than that from either stem or leaves. More than 98% of 2-instar larvae were killed when feeding on 1-3-mL QRFE treated diet. It seems that phytoecdysteroids contained in the root is more than in other organs.

Those extracts made from plants collected from different spots did not show significant difference in the pesticide ability to the 2-instar larva of *C. lapathi* L, even though the climate condition are quit different between those spots. From our experiment it was impossible to affirm if the contentment of phytoecdysteroids was different between those plants produced at Qianshan, Zaliantun or Jigedaqi. It is commonly believed that the differences in clone, strain, living condition, *etc.* may influence the contentment of phytoecdysones in the plants and further influence the pesticides ability of those plants (Tomas *et al.* 1992).

Two phenomena were found in this experiment. One was that the killing speed of extracts from *A. multiflora* on the larvae of *C. lapathi* L. was slower than that of other pesticides treated groups. The mortality rate of those larvae was not higher than 50% until the time feeding on the treated diet for 16 days. The other pesticides used in this experiment such as HEC, TEC or DEC has a faster killing speed. The other phenomena were that the time period of feeding on the treated diet affects the mortality rate significantly. When larvae fed on the treated diet less than 12 d, after then their diet was changed to the untreated one, the death rate of those larvae were very low. Only after feeding on the treated diet more than 16 d, the mortality rate of larvae increased rapidly. Those results were probably attributed to specially acting mechanism of those extracts or the special biological characteristic of the larvae. The main compounds extracted from *Ajuga* plant are phytoecdysone analogs. Those compounds mainly work on the time when insects are molting or metamorphism. The growing period of 2-instar larvae was about 20 d. At the beginning of the treatment those larvae might not reach their molting time so those phytoecdysones did not work. The other possibility was that those larvae did not accumulated enough amount of phytoecdysones until fed on the treated diet for 16 days, so they were still living. The exact reasons of these phenomena need further study.

In general consideration, phytoecdysteroids was unstable under high temperature. We added the powder and extracts of *Ajuga* into the artificial diet before it was steamed, and we used the steamed diet with added extracts as contrast. All those diets showed killing effect on those treated larvae, and there were no significant difference in the mortality rates between those diets. The mortality rate of those larvae fed on the diets added with 1-mL (g) phytoecdysteroids was 66.67%, 68.79%, and 64.15% by treating methods of MQWFE, SQWFE and MQWFP, respectively. This means that, at least, those compounds led the 2-instar larvae to death did not invalidated by

steaming.

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